Exercise Prevents Western-Diet Associated Erectile Dysfunction and Coronary Artery Endothelial Dysfunction: Response to Acute Apocynin and Sepiapterin Treatment

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\textbf{Running Head:} Exercise prevention of erectile and coronary dysfunction

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ABSTRACT

The aim of this study was to investigate aerobic exercise training as a means to prevent erectile dysfunction (ED) and coronary artery disease (CAD) development associated with inactivity and diet-induced obesity. Male Sprague-Dawley rats were fed a Western diet (WD) or Control diet (CD) for 12 weeks. Subgroups within each diet remained sedentary (Sed) or participated in aerobic interval treadmill running throughout the dietary intervention. Erectile function was evaluated under anesthesia by measuring the mean arterial pressure and intracavernosal pressure in response to electrical field stimulation of the cavernosal nerve, in the absence or presence of either apocynin, an NADPH oxidase inhibitor, or sepiapterin, a tetrahydrobiopterin precursor. Coronary artery endothelial function (CAEF) was evaluated ex vivo with cumulative doses of acetylcholine applied to pre-constricted segments of the left anterior descending coronary artery. CAEF was assessed in the absence or presence of apocynin or sepiapterin. Erectile function (P < 0.0001) and CAEF (P < 0.001) were attenuated in WD-Sed. Exercise preserved erectile function (P < 0.0001) and CAEF (P < 0.05) within the WD. Erectile function (P < 0.01) and CAEF (P < 0.05) were augmented by apocynin only in WD-Sed, while sepiapterin (P < 0.05) only augmented erectile function in WD-Sed. These data demonstrate that a chronic WD induces impairment in erectile function and CAEF that are commonly partially reversible by apocynin, whereas sepiapterin treatment exerted differential functional effects between the two vascular beds. Furthermore, exercise training may be a practical means of preventing diet-induced ED and CAD development.

Key words: Diet-Induced Obesity, NADPH oxidase, high-fat high-sucrose, eNOS uncoupling, high-intensity interval training
INTRODUCTION

Obesity is no longer strictly an American problem. Obesity rates have drastically risen in much of the developed world since the 1980’s, and continue to rise in much of the developing world (16). Corresponding to these trends is the increasing worldwide consumption of the Western diet (WD), consisting of high levels of saturated fat, omega-6 (n-6) polyunsaturated fatty acids (PUFA), particularly n-6 linoleic acid, as well as added sugar (46). Obesity has proven to be a significant, independent risk factor for erectile dysfunction (ED), thus the prevalence of ED is projected to rise alongside the rising obesity rates (5, 30). Furthermore, clinical data suggests that presence of ED in otherwise healthy men may be associated with early, subclinical signs of coronary artery disease (CAD) that may not be detectable during stress testing (8, 31, 40). A recent meta-analysis of 92,757 subjects followed for a mean of 6.1 years confers that patients with ED have a 62% increased risk for myocardial infarction compared to patients without ED, which is independent of traditional cardiovascular risk factors (61). We have recently demonstrated that rats fed a WD develop ED prior to coronary artery endothelial dysfunction (36). Thus, ED may be a powerful predictor of CAD and therapeutic strategies aimed at treatment of both of these diseases warrants investigation.

Oxidative stress, marked by excessive production of reactive oxygen species (ROS), is thought to be elevated in obese individuals and is a prominent feature of vascular diseases (9, 19). NADPH oxidase (NOX), in particular, is considered a prominent source of vascular derived ROS, the expression of which has shown to be elevated in arteries of human diabetes and CAD patients (22, 23). A potential consequence of increased NOX activity is oxidation of tetrahydrobiopterin (BH₄), an essential cofactor of endothelial nitric oxide synthase (eNOS) (11). When BH₄ is deficient eNOS dimers destabilize, resulting in formation of superoxide rather than
nitric oxide (NO) (11). Production of superoxide from eNOS is termed uncoupling, which represents a potential vicious cycle of vascular dysfunction by promoting depressed NO bioavailability and increased oxidative stress through excessive superoxide production. Neuronal nitric oxide synthase (nNOS) may also uncouple by a similar mechanism (49), which has recently been shown to potentiate endothelial dysfunction in penile arteries (48).

Exercise training is known to protect against cardiovascular disease (35). Aerobic interval training in particular has shown to produce several positive health benefits in patients with heart failure or the metabolic syndrome, including increases in aerobic capacity, flow-mediated dilation, insulin sensitivity, and decreases in fatty acid synthase (56, 66). The purpose of this study was to determine if aerobic interval training could protect against WD-induced ED and coronary artery endothelial dysfunction. Further, we sought to investigate the efficacy of acute apocynin (NOX1/2 inhibitor) and sepiapterin (BH₄ precursor) treatment on erectile function and CAEF in rats fed a WD.
METHODS

Experimental Animals and Diets

Male Sprague-Dawley rats were purchased at 5 weeks of age (Charles River Laboratories, Wilmington, MA) and housed in the Department of Comparative Medicine at East Carolina University, in pairs when possible, in a temperature controlled room (22 ± 1°C) with a 12h:12h light dark cycle. Rats were fed a high fat-high sucrose diet with a Western pattern fatty acid distribution (Teklad Diets 110365, Harlan Laboratories, Madison, WI) (WD) or a control diet (Teklad Diets 110367) (CD) for the final 12 weeks of life. The Western diet (4.68 kcal/g, 44.6 %kcal fat, 40.7% kcal carbohydrate, 340 g/kg sucrose, 27 n-6:n-3 ratio) and control diet (3.67 kcal/g, 12.7 %kcal fat, 72.4 %kcal carbohydrate, 150 g/kg sucrose, 9.6 n-6:n-3 ratio) contained equivalent levels of vitamins, minerals, and protein when considered on a basis of kcal density, as further described (36). All rats were sacrificed at 19-20 weeks of age. All procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals established by the National Institute of Health and approved by the Institutional Animal Care and Use Committee at East Carolina University.

Exercise Intervention

Rats remained sedentary (n = 12 CD, n = 11 WD) or underwent an aerobic interval training intervention (n = 8 CD, n = 11 WD) throughout the duration of the dietary intervention. Rats were acclimated to exercise by walking on a treadmill at 15 m/min at 0° inclination for 10 min/day for seven consecutive days prior to induction of the training/dietary intervention. The training protocol consisted of a maximal work capacity test at the beginning of each week, followed by aerobic interval training four days/week, for a total of five exercise days/week. The
maximal work capacity test consisted of a 20-minute warm-up at ~50% VO₂max, followed by incremental increases in speed of 2.0 m/min every two minutes until volitional fatigue, followed by a 10-minute cool-down at ~50% VO₂max. The aerobic interval protocol consisted of a 10-minute warm-up followed by eight intervals, each consisting of a four-minute high-intensity phase and three-minute low-intensity phase, followed by a four-minute cool-down as detailed in Table 1. Running intensity increased each week until a plateau was reached in maximal work capacity at week 9. Rats were encouraged to run primarily by compressed air, with every effort made to minimize the use of the electric shock grid at the back of the treadmill belt. This protocol was designed to approximate the intensities utilized in previous studies which demonstrate the beneficial impact of aerobic interval training on endothelial function and aerobic capacity relative to continuous moderate intensity exercise protocols (25, 26, 28, 65).

**Glucose, insulin, lipids and anthropometrics**

Approximately 0.3 ml of blood was drawn from the tail vein prior to anesthesia, from which fasting glucose concentration was measured from whole blood (Accu-Check, Roche, Basel, Switzerland). Plasma was separated and stored at -80°C until analysis of insulin with a rat/mouse insulin assay kit (Millipore, Billerica, MA). Serum was collected during the erection surgery as described (36), separated and stored at -80°C until analysis. Total cholesterol, high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), and triglycerides were measured with a clinical mass analyzer (UniCel DxC 600, Beckman Coulter, Indianapolis, IN). Serum 4-hydroxynonenal (HNE) adducts were measured with a modified ELISA approach as described previously (17). Body weight was measured prior to surgery with a triple beam balance. Lean mass and fat mass were measured immediately prior to surgery by
nuclear magnetic resonance-magnetic resonance imaging (NMR-MRI) (EchoMRI 700, Echo
Medical Systems, Houston, TX).

**Erectile Response Measurements**

All rats were fasted overnight prior to erectile function studies. Rats were anesthetized
with an intraperitoneal (IP) injection of 90 mg/kg ketamine and 10 mg/kg xylazine, and
supplemented with an intramuscular injection as needed. The left carotid artery was cannulated
with a 20-gauge needle connected via polyethylene (PE) tubing to a pressure transducer, anti-
coagulated with 2.0 U/ml heparinized saline allowing for continuous measurement of systemic
blood pressure and intra-arterial administration of saline. The right corpus cavernosum was
cannulated with a 27-gauge needle connected via PE tubing to a pressure transducer, and the left
corpus cavernosum cannulated with a 27-gauge needle connected via PE tubing to a syringe.
Platinum bipolar electrodes attached to a Grass Stimulator (Grass S09, Grass Technologies, West
Warwick, RI) were positioned near the cavernosal nerve to deliver electrical field stimulation,
consisting of a successive, increasing voltage series of 1-5 volts for 30 seconds at each voltage,
delivered in 5-millisecond pulses at 13 Hz (36). Mean arterial pressure (MAP) and
intracavernosal pressure (ICP) were recorded throughout each voltage series with LabChart 7
software (ADInstruments, Sydney, Australia). Three voltage series’ were conducted and
averaged as the baseline erectile response. ICP returned to baseline between each series. We
have found the erectile response to these stimulation parameters to be stable for at least two
hours. Following the three voltage series’, either 12 µl of 1 mM apocynin (Sigma Aldrich, St.
Louis, MO) or 12 µl of 10 µM sepiapterin (Cayman Chemical, Ann Arbor, MI), prepared in
heparinized saline, was injected into the left corpus cavernosum. A voltage series was conducted
20-minutes post-apocynin injection or 30-minutes post-sepiapterin injection, where treatment effects appeared to be maximal.

**Coronary Artery Endothelium-Dependent and -Independent Vasorelaxation**

Immediately following the erection surgery, rats were euthanized by exsanguination and double pneumothorax. Hearts were excised and up to four segments of the left anterior descending (LAD) coronary artery were carefully dissected and freed from adhering myocardium. Two, 40 µm diameter, stainless steel wires were passed through each arterial segment and mounted in a multi wire myograph (DMT 620 M, Aarhus, Denmark). Vessel segments were bathed in physiological saline solution (PSS) at pH 7.4, 37°C, gassed with medical grade air as described (64). Vasorelaxation assessments were performed as described (36). Briefly, vessels were gradually stretched to an optimum resting tension, established by determining diameter-tension relationship of each segment and setting it to 90% of the lumen circumference achieved at 13.3 kPa, and viability was tested with a 109 mM K⁺ physiological saline solution (KPSS) challenge. Vessels that failed to constrict to at least 1.0 mN/mm² were excluded from experimentation. Following 30 minutes of repeated washes of PSS at 10-minute intervals, vessels were pre-constricted with 3.0 µM 5-hydroxytryptamine (5-HT), and endothelial function tested with cumulative concentrations of acetylcholine (ACh) (0.001 – 10.0 µM). Following 30 minutes of repeated washes of PSS at 10-minute intervals, vessel segments were incubated in either 300 µM apocynin or 10 µM sepiapterin for 30 minutes, and the ACh cumulative concentration response following 5-HT pre-constriction was repeated. Vessels were washed for 30 minutes with repeated washes of PSS at 10-minute intervals, and endothelium-independent relaxation was tested with cumulative concentrations of sodium nitroprusside (SNP) (0.0001 – 1.0 µM) following 5-HT pre-constriction.
Aortic NADPH Oxidase Activity and TBARS

Following excision of the heart, the thoracic aorta was excised and placed in ice-cold PSS. Adhering connective tissue and adipose tissue were removed. Aortas were snap frozen in liquid nitrogen and stored at -80°C until analysis. Aortas were homogenized in 10X (wt./vol) TEE buffer (10 mM Tris base, 1 mM EDTA, and 1 mM EGTA) using a tissue grinder (Kimble Chase, Vineland, NJ). 0.1% Triton-X was added following homogenization. Each homogenate was divided into two aliquots. One aliquot was centrifuged at 1,600 x g for 10 minutes at 4°C, the supernatant separated and stored on ice until measurement of NOX activity. For this assay, 30 µl of sample was added to 170 µl of a cocktail containing 10 µM Amplex Red (Invitrogen, Eugene, OR), 1.0 U/ml horseradish peroxidase (Sigma Aldrich, St. Louis, MO) and 10 U/ml superoxide dismutase (Sigma Aldrich, St. Louis, MO) in PBS. Fluorescence of resorufin (563 ex/587 em) was measured continuously with a spectrofluorometer (Horiba Jobin Yvon, Ann Arbor, MI) with temperature control and magnetic stirring at 37°C (3). After establishing baseline rate of $\Delta F/dt$ in the absence of substrate, NOX activity was stimulated with addition of 500 µM NADPH, following which $\Delta F/dt$ was continuously measured for ~5 minutes. Total tissue NOX activity was calculated as $\Delta F/dt$ of resorufin, where total NADPH-dependent H$_2$O$_2$ generated in the sample was used as an index of NOX activity. Activity was normalized to total protein content determined by a bicinchoninic acid (BCA) assay (Pierce Biotechnology, Rockford, IL).

The other aliquot was sonicated for 15 seconds at 40V (Branson sonifer 450) over ice, centrifuged, and the supernatant was stored at -80°C until analysis of thiobarbituric acid reactive substances (TBARS), an index of lipid peroxidation and associated oxidative stress. TBARS were measured in duplicate with a commercial assay kit (Cayman Chemical, Ann Arbor, MI).
according to the manufacturer’s instructions. Protein concentrations from these samples were
determined using a BCA assay kit. Sample malondialdehyde (MDA) concentration was
calculated from a MDA standard curve, and concentration was normalized to total protein
content.

**Data and Statistical Analysis**

The erectile response was calculated from the ratio of the average ICP divided by the
average MAP during the final 25 seconds of each 30-second voltage stimulation level. Effects of
acute apocynin or sepiapterin treatment on the erectile response were compared to the pre-
treatment response within the same rat. Coronary artery endothelium-dependent and -
independent vasodilation was assessed by the average stress produced by each vessel in the final
30 seconds of each 5-minute concentration exposure. Vessel stress was calculated by subtracting
basal vessel force produced from the force produced at each respective concentration and
normalized to vessel surface area. The relaxation responses from the vessel segments were
averaged for each rat, and analyzed as a single observation. The effects of apocynin or
sepiapterin treatment were compared to the untreated response within the same segment.
Concentration response curves were generated against the logarithm of each concentration, and
fit by nonlinear regression to generate EC$_{50}$ values to describe each curve (GraphPad Prism v.
5.0). E$_{\text{max}}$ values were determined as the maximal relaxation of each segment, expressed as a
percentage of 5-HT induced constriction. Statistical differences for the voltage-dependent
erectile response and coronary artery agonist-stimulated vasorelaxation were determined by two-
way repeated measures ANOVA with Bonferroni’s post-hoc analysis. Between group differences
for EC$_{50}$ and E$_{\text{max}}$ values were determined by one-way ANOVA with Tukey’s multiple
comparisons post-hoc analysis. Treatment effects of apocynin or sepiapterin on EC$_{50}$ and E$_{\text{max}}$
values were determined by a paired t-test. Statistical differences for metabolic parameters, NADPH oxidase activity, and TBARS were determined by one-way ANOVA with Tukey’s multiple comparisons post-hoc analysis. Data were presented as mean ± SEM. Statistical analyses were performed with GraphPad Prism v. 5.0 or SPSS v. 19.0, with an alpha level of 0.05.
RESULTS

Metabolic Parameters

The body weight from the CD-Ex rats was lower than WD-Sed; however WD-Sed rats had greater fat mass than all other groups while lean mass was not different among any group (Table 2). Despite increased blood glucose in the WD-Sed rats, no differences in insulin or HOMA-IR (Table 2) were observed between groups. WD-Ex rats had lower total cholesterol levels than CD-Sed rats, while no differences existed for HDL-cholesterol, LDL-cholesterol, or triglyceride levels between groups (Table 2). Systemic oxidative stress was lower in both exercise groups compared to the WD-Sed group, as indicated by serum 4-hydroxynonenal (HNE-adducts; Table 2).

Voltage-Dependent Erectile Response

The ICP/MAP in response to the intermediate voltages (2, 3, and 4V) was significantly attenuated by the Western diet (Figure 1A), and was preserved by exercise training within the Western diet (Figure 1C). However, the ICP/MAP at 2 and 3V was significantly attenuated by exercise training within the control diet (Figure 1D). Intracavernosal treatment with apocynin (Figure 2) or sepiapterin (Figure 3) had no effect on the voltage-dependent erectile response of the CD-Sed, CD-Ex, or WD-Ex rats, but significantly augmented the erectile response of WD-Sed rats.

Coronary Artery Endothelium-Dependent and -Independent Relaxation

Vasoconstriction tested by KPSS challenge (P = 0.828) or 3.0 µM 5-HT (P = 0.945) were not different between any groups (Table 3). The ACh-stimulated relaxation curves are presented
in Figure 4. The relaxation profile was significantly attenuated by the WD at two ACh concentrations (Figure 4A). Similarly, exercise augmented the relaxation profile within the WD at two ACh concentrations (Figure 4C). Corresponding relaxation profile analysis reveals an greater EC$_{50}$ value and depressed E$_{max}$ response to ACh in the WD-Sed group (Table 3). Treatment of the vessel segments with apocynin augmented ACh-stimulated vasorelaxation profile in WD-Sed, but had no significant effect on CD-Sed, Con-Ex, or WD-Ex coronary artery segments (Figure 5). The effect of apocynin treatment on EC$_{50}$ ($P = 0.065$) and E$_{max}$ ($P = 0.079$) failed to reach statistical significance (Table 3). Treatment with sepiapterin did not improve ACh-stimulated vasorelaxation in any group, while sepiapterin attenuated the vasorelaxation profile, EC$_{50}$, and E$_{max}$ in CD-Sed (Figure 6, Table 3), and E$_{max}$ in WD-Ex (Table 3). The coronary artery endothelium-independent relaxation was assessed from the concentration-response profile to the NO donor sodium nitroprusside. There were no significant differences between groups for the relaxation response to SNP (Figure 7); however the E$_{max}$ was greater in the WD-Sed group compared to the CD-Ex group (Table 3).

**Vascular Tissue Oxidative Stress**

All attainable LAD tissue was utilized for the myography experiments. Use of cavernosal tissue to perform NOX activity and oxidative stress measurements was untenable following apocynin/sepiapterin treatment; therefore the thoracic aorta was utilized as a surrogate vascular tissue to investigate these parameters. NADPH stimulated ROS production was elevated in the WD-Sed group compared to both the CD-Sed and WD-Ex groups (Figure 8A). Aortic TBARS was measured as an index of vascular tissue lipid peroxidation (Figure 8B), where there were no statistically significant differences between groups ($P = 0.227$).
DISCUSSION

In the present study, we investigated the efficacy of an aerobic interval training intervention on prevention of WD-induced ED and coronary artery endothelial dysfunction. Furthermore, we investigated the differential effects of acute apocynin treatment and acute sepiapterin treatment on erectile function and CAEF following a 12 week WD that is high in sucrose and fat derived from saturated and n-6 PUFA. Rats demonstrated an impaired erectile response to electrical field stimulation of the cavernosal nerve, and an impaired coronary artery relaxation response to ACh following the chronic WD, both of which were prevented by the exercise intervention. Acute apocynin treatment significantly improved erectile function and CAEF in the WD-Sed group, while having no impact in the CD or Ex groups. In contrast, stimulation of intracellular BH₄ production with acute sepiapterin treatment significantly improved erectile function in the WD-Sed group, but had no effect on CAEF. Acute sepiapterin treatment attenuated CAEF in the CD-Sed and WD-Ex group, while having no effect on the WD-Sed group. These findings suggest that erectile function and CAEF are impaired by commonly a mechanism which may be targeted by acute apocynin treatment in a 12 week WD-induced obesity model. However, despite the common functional improvement mediated by apocynin treatment, the two vascular beds demonstrated a differential functional response to BH₄ production stimulation with sepiapterin.

Oxidative stress has previously been demonstrated in cavernosal tissue of LDLR⁻/⁻ mice fed a hypercholesterolemic diet (42), cholesterol fed rabbits (50), and high-fat diet fed pigs (43). Oxidative stress is a likely contributor to ED, as treatment with various antioxidants (4, 53) or transfection of SOD (7) normalize markers of cavernosal oxidative stress and augments cavernosal vasorelaxation in STZ-diabetic rats. Mitochondria-derived oxidative stress may be a
pathological consequence of the metabolic syndrome that contributes to vascular dysfunction (63), however the impact of mitochondria-derived ROS on erectile function remains unknown. Cytosolic derived ROS have more traditionally been associated with erectile dysfunction, as elevated expression of NOX subunits have been found in cavernosal tissue of STZ-induced diabetic rats (38) and LDLR⁻/⁻ mice fed a hypercholesterolemic diet (42). Additionally, chronic supplementation of apocynin to drinking water prevents hypercholesterolemic diet (42) and hypertension (33) associated vascular insult and preserves the voltage dependent erectile response. The present study suggests that an apocynin targeted mechanism partially reverses WD associated ED. This result an important distinction from a chronic supplementation study, as we postulate that acute apocynin treatment only restores functionality, presumably by inhibiting NOX1/2 derived ROS production, whereas chronic apocynin supplementation may also inhibit progressive atherosclerotic development and vascular remodeling resulting from chronic oxidative stress.

One potentially vasodestructive consequence of elevated NOX activity is the quenching of NO by superoxide, resulting in the production of peroxynitrite. Further, peroxynitrite may then deplete intracellular BH₄ levels, by direct oxidation of BH₄ to BH₂ (37). Adequate presence of BH₄ is a critical requirement for normal eNOS functioning, which acts to stabilize eNOS dimers and allows for the oxidation of L-arginine and the subsequent generation of NO (11). Destabilization of eNOS dimers through BH₄ depletion results in “eNOS uncoupling”, where electron flow from NADPH through eNOS to O₂ is not inhibited, but results in the formation of superoxide rather than NO (11). The resulting situation is a potential vicious cycle where NOX activity is elevated in metabolic syndrome conditions, which promotes endothelial dysfunction through NO quenching and eNOS uncoupling, and further endothelial dysfunction via
progressive loss of NO bioavailability and superoxide production from eNOS. LDLR−/− mice fed a hypercholesterolemic diet demonstrate a decreased cavernosal eNOS dimer:monomer ratio, which is prevented by chronic drinking water supplementation of apocynin (42). Although nNOS uncoupling is studied much less than eNOS uncoupling, nNOS may become uncoupled via BH4 oxidation (49). This may be a particularly important mechanism regulating erectile function, as nNOS-derived NO is critical for the initiation of erection (47). The concept of nNOS uncoupling mediating ED has recently been brought to light by Sánchez et al (48), who demonstrated improved nitrergic vasorelaxation of penile arteries in response to BH4 or apocynin in a genetically obese rat model. In the present study, we observed a partial reversal of obesity associated ED in response to acute BH4 production stimulation with sepiapterin. While we cannot be certain that NOX was the predominant mechanism by which cavernosal NOS was apparently dysfunctional in response to the WD, apocynin treatment and BH4 production stimulation with sepiapterin both augmented the voltage-dependent erectile response to a similar degree.

Contrary to the voltage-dependent erectile response, acute sepiapterin treatment did not improve coronary artery endothelial dysfunction in the WD-Sed rats. 10 µM sepiapterin treatment has previously restored endothelial function in aortic segments of ApoE−/− mice (37). Furthermore, oral administration of sepiapterin has been found to augment left ventricular function in STZ-induced diabetic mice (34), while intravenous administration of sepiapterin restores the cardioprotective effect of ischemic preconditioning that is completely blocked by severely hyperglycemic conditions (62). However, investigations on the effect of sepiapterin treatment specifically on CAEF have been limited. 100 µM sepiapterin has previously been found to attenuate endothelial dependent vasorelaxation responses in canine middle cerebral
arteries (57) and rabbit aortas (58), which agrees with the findings of the present study in which 10 µM sepiapterin attenuated ACh-stimulated vasorelaxation in coronary artery segments of rats with preserved endothelial function. It has previously been suggested that sepiapterin supplementation to normally functional eNOS could stimulate eNOS-dependent superoxide generation, resulting in potential for attenuation of endothelial function (59). Additionally, the hypothesis of NOX induced eNOS uncoupling suggests that NOX activity is increased prior to any depletion of BH₄ and resultant eNOS uncoupling (2). The WD used in the present study has previously been demonstrated to induce ED more rapidly than coronary artery endothelial dysfunction (36). Thus, it is probable that NOX activity is also increased in the cavernosum prior to the LAD coronary artery. It is likely that 12 weeks of exposure to the WD represents a very early time point in development of CAD in this rat model (36). These WD-Sed rats demonstrated mild elevations in adiposity and blood glucose levels, with no substantial alterations in blood lipids or insulin sensitivity. Thus, it is possible that prolonging the dietary intervention may deplete intracellular BH₄ levels and induce NOS uncoupling in the coronary vasculature, as prior studies demonstrating increased vasoprotection or cardioprotection with sepiapterin treatment have utilized overtly hypercholesterolemic, hyperglycemic, or diabetic models (34, 37, 62). This study is in agreement with prior investigations, where apocynin has been used by the Zhang group (20, 44, 45) to reverse endothelial dysfunction in coronary microvessels of insulin resistant obese Zucker rats (45), type II diabetic mice (20), and high-fat diet induced obese mice (44). This study suggests that elevated NOX activity is an important contributor to early WD-associated coronary artery endothelial dysfunction, where functionality is not restored with BH₄ production stimulation.
The potential of lifestyle interventions including dietary and/or physical activity alterations on treating ED has been discussed in several review articles (14, 21, 24, 39). Across general populations of men, physical activity is generally associated with better erectile function (13, 29, 32). However, investigations on the impact of exercise interventions on obesity associated erectile dysfunction are rather limited. In a groundbreaking study by Esposito et al. (15), obese men with ED who ate a caloric restricted diet for two years and received advice to increase physical activity reported reduced severity of erectile dysfunction coinciding with substantial weight loss. While the present study fails to address the question of whether exercise is exclusively capable of reversing ED, exercise completely preserved erectile function despite ad libitum access to the obesogenic WD which rapidly induces ED (36). There are previous implications for a protective effect of exercise through eNOS coupling, as treadmill exercise of pigs has shown to preserve the cavernosal eNOS dimer:monomer ratio that is attenuated in response to a hypercholesterolemic diet (43). The present study adds to these findings, as exercise preserves erectile function despite chronic WD consumption, whereby chronic WD consumption induced impaired erectile functionality that was partially reversible with BH4 production stimulation. An unexpected finding from this study was that exercise attenuated erectile function within the control diet. The exercise protocol was challenging, which could create undesirable stress for the rats. High-volume, high-intensity exercise training has previously been found to attenuate peripheral vascular function in lean humans (6) and aortic endothelial function in lean rats (52). However, WD-Ex rats were subjected to the same stress that CD-Ex were, which highlights the protective effects of exercise in the WD condition. Failure of apocynin to alter erectile function in either exercised condition may suggest that NOX
activity was not upregulated in the cavernosum, as was demonstrated in the aorta and supported by low levels of systemic oxidative stress measured by serum HNE-adducts.

Treadmill exercise has previously proven to preserve CAEF through a NO-mediated mechanism in a hypercholesterolemic pig model (55). Importantly, exercise has shown to improve endothelial function in internal mammary artery segments and attenuate NOX subunit expression in CAD patients (1). Similar to the present study, the apocynin-induced augmentation of CAEF in high-fat diet fed mice is lost with the addition of voluntary wheel running (44). Protection from ROS provided by the endogenous antioxidant system is critical to maintenance of redox balance and protection from vascular insult, however the response of antioxidants to diet, exercise, and pathologies have been variable. Penile SOD expression is decreased (53), while SOD activity is not changed with STZ-induced diabetes (7). SOD-1 expression does not change with high-fat diet in mesenteric arteries, but increases with exercise irrespective of diet (12). SOD-1 expression is depressed with high-fat diet in coronary arteries (44, 55), while SOD-1 increases (44) or does not change (55) with exercise training. Gene expression of an array of antioxidant genes were upregulated with WD and/or Ex in myocardial tissue from the rats in the present study (17), which may promote a cardioprotective phenotype at the time point in which these rats were studied. Transient, exercise induced ROS production induces a hormesis effect, whereby increased ROS production provides a signal to stimulate the antioxidant defense system (18, 67). Exercise induced cardioprotection has been shown to be strongly associated with myocardial SOD activity (67). Interestingly, it was recently reported that NOX derived ROS are responsible for exercise induced cardioprotection as IP injection of apocynin prior to exercise prevented cardioprotection, whereas IP injection of mitochondrial targeted ROS scavengers were ineffective (18). These findings are exciting; however the
implications for transient exercise-induced NOX activation in pathological states where NOX activity is constitutively elevated remain a fascinating area of future investigation.

Aerobic interval training may be a particularly advantageous mode of intervention in the metabolic syndrome. Interval training protocols similar to that utilized in the present study have been shown to substantially augment endothelial function and aerobic capacity in human metabolic syndrome patients (56), heart failure patients (66), and CAD patients (41), as well as rodents bred to have a low aerobic capacity (26). The present study demonstrates that aerobic interval training is an effective method for preserving sexual function and CAEF despite consumption of an obesogenic diet which promotes vascular dysfunction.

Several limitations exist to the present study. Apocynin is classically reported to inhibit the assembly of p47phox and p67phox within the membrane complex, and thus inhibit activation of NOX isoforms that require subunit translocation (51). However, apocynin has been reported to be both an antioxidant (27) and a pro-oxidant (10). Thus, we cannot be certain that apocynin acted specifically to inhibit NOX activity in our model. Furthermore, we demonstrated elevated NOX activity in aortas of the WD-Sed group which support our functional data with apocynin treatment; however we cannot be certain that NOX activity of the aorta translates to the cavernosum or the coronary artery. Also, BH4 may act as an antioxidant (11). Thus, in the absence of NOS dimer:monomer measurements, we cannot be certain that sepiapterin induces NOS coupling rather than oxidant scavenging. However, apocynin and sepiapterin elicited similar treatment effects on erectile function, but differing effects on CAEF. Thus, it is unlikely that both agents are acting merely as antioxidants. It should also be noted that treatment effects were compared to untreated responses within the same rat or same vessel segment. There was no vehicle treated group in these experiments to assess the effect of time on responsiveness because
the vehicles was our PSS solution and all drugs were PSS soluble. We have previously performed parallel tissue experiments to assure reproducibility of the vessel responses and erectile responses over time and found no significant diminution in repeated responses over the time course in which these studies were carried out. We acknowledge that without a specific vehicle treated group in these experiments it remains possible that a time effect may have masked potential effects of apocynin in the exercised and CD-Sed group. Additionally, the study design utilizing an exercise intervention initiated at the onset of the dietary intervention does not allow one to infer if the exercise training effects observed with the WD are merely due to the prevention of obesity or a more complex interaction between diet and exercise.

PERSPECTIVES AND SIGNIFICANCE

The findings of this study show that rats fed 12 weeks of a high-fat, high-sucrose diet with a Western pattern fatty acid distribution demonstrate ED and coronary artery endothelial dysfunction. Dysfunctionality of both vascular beds was partially reversed by acute apocynin treatment. Despite the common effect of apocynin treatment, the two vascular beds demonstrated a differential response to acute sepiapterin treatment. Furthermore, these dysfunctions are preventable with concomitant aerobic interval exercise training. In the face of a worldwide obesity pandemic resulting from global spread of the Western diet (46), novel approaches to treatment and prevention of CAD are increasingly necessary. The association between ED and CAD has been shown to be nearly identical to the risk associated with current smoking or family history of myocardial infarction (54). This study demonstrates common efficacy of acute, localized pharmacologic treatment of ED and CAD at a time point that likely translates to a progressed stage of ED and a very early stage of development of CAD (36). These findings come at the heels of a recent review discussing clinical ED management
strategies for patients with varying degrees of CVD risk, that also calls for sexual health
counseling of CVD patients (60). The finding that exercise prevents WD-associated ED and
CAD progression translates to an intensively active lifestyle throughout the duration of the “junk
food” diet. It remains to be seen if a moderately active lifestyle, or an active lifestyle initiated
after a prolonged duration of a sedentary lifestyle combined with a “junk food” diet is effective
at reversing functional impairment. While presentation of ED represents an attractive time point
for lifestyle and/or therapeutic intervention aimed at cardioprotection, future studies are
necessary to establish these relationships.
REFERENCES


ACKNOWLEDGEMENTS

We would like to thank Kathleen Thayne for assistance with phlebotomy, and Mike McCammon for the use of mass analyzer reagents.

GRANTS

This work was supported by a Trainee grant from the Sexual Medicine Society of North America (to J.D.L)

DISCLOSURES

The authors declare no conflict of interest, financial or otherwise.

AUTHOR CONTRIBUTIONS

J.D.L. contributed to conception and design of the experiments, collection, analysis, and interpretation of data, and drafting the manuscript. E.J.A. contributed to conception and design of the experiments and critically revising the manuscript. J.T.D. contributed to collection of data. R.C.H. contributed to conception and design of the experiments and critically revising the manuscript. C.J.W. contributed to conception and design of the experiments, analysis and interpretation of data, and critically revising the manuscript.
TABLE CAPTIONS

Table 1 Treadmill aerobic interval training protocol. Treadmill speed was changed at the indicated time point.

Table 2 Metabolic Parameters. Values are means ± SEM, N = 5-11 animals per group. * P < 0.05 vs. CD-Sed, † P < 0.05 vs. CD-Ex, ‡ P < 0.05 vs. WD-Ex. CD, Control diet; WD, Western diet; Sed, Sedentary; Ex, Exercise.

Table 3 Coronary artery vasoconstriction and vasorelaxation characteristics. Values are means ± SEM, N = 6-11 animals per group. * P < 0.05 vs. CD-Sed, § P < 0.05 vs. CD-Ex, † Significant treatment effect. CD, Control diet; WD, Western diet, Sed, Sedentary; Ex, Exercise; ACh, acetylcholine; SNP, sodium nitroprusside; Apo, apocynin; Sep, sepiapterin.
FIGURE CAPTIONS

**Figure 1** Voltage-dependent erectile responses. ICP/MAP was attenuated by the WD (A). ICP/MAP was equivalent with exercise between diets (B). WD-induced impairment of ICP/MAP was prevented by exercise training (C). ICP/MAP was attenuated by exercise training within the control diet (D). Reported are means ± SEM for 8-11 animals in each group; * P < 0.05, ** P < 0.01, **** P < 0.0001. ICP, intracavernosal pressure; MAP, mean arterial pressure; CD, control diet; WD, Western diet; Ex, exercise; Sed, sedentary.

**Figure 2** Effect of acute apocynin administration on voltage-dependent erectile response. Following the untreated voltage series’ (open bars), 1 mM apocynin was injected intracavernosally and a voltage series was applied 20-minutes post-injection (hatched bars). No treatment effects were observed in control diet-sedentary (CD-Sed) (A), control diet-exercise (CD-Ex) (B), or Western diet-exercise (WD-Ex) (D), while apocynin enhanced the erectile response in Western diet-sedentary (WD-Sed) rats (C). Reported are means ± SEM for 4-5 animals in each group ** P < 0.01 vs. untreated. ICP, intracavernosal pressure; MAP, mean arterial pressure.

**Figure 3** Effect of acute sepiapterin administration on voltage-dependent erectile response. Following the untreated voltage series’ (open bars), 10 µM sepiapterin was injected intracavernosally and a voltage series was applied 30-minutes post-injection (checkered bars). No treatment effects were observed in control diet-sedentary (CD-Sed) (A), control diet-exercise (CD-Ex) (B), or Western diet-exercise (WD-Ex) (D), while sepiapterin enhanced the erectile response in Western diet-sedentary (WD-Sed) rats (C). Reported are means ± SEM for 4-6
animals in each group * P < 0.05 vs. untreated. ICP, intracavernosal pressure; MAP, mean arterial pressure.

**Figure 4** Mean concentration-response of coronary artery segments following 3.0 µM 5-HT pre-constriction to acetylcholine (ACh) stimulation (0.001 – 10.0 µM). ACh-stimulated vasorelaxation was impaired by the Western diet (A), and equivalent with exercise between diets (B). WD-induced impairment of ACh-stimulated vasorelaxation was prevented by exercise training (C), while exercise training had no effect within the control diet (D). Reported are means ± SEM for 6-11 animals in each group. * P < 0.05, *** P < 0.001. CD, control diet; WD, Western diet; Ex, exercise; Sed, sedentary.

**Figure 5** Effect of apocynin on acetylcholine (ACh)-stimulated vasorelaxation of coronary artery segments. Vasorelaxation was examined in the absence (closed circles) and presence (open circles) of 300 µM apocynin. No treatment effects were observed in control diet-sedentary (CD-Sed) (A), control diet-exercise (CD-Ex) (B), or Western diet-exercise (WD-Ex) (D), while apocynin enhanced ACh-stimulated vasorelaxation in Western diet-sedentary (WD-Sed) rats (C). Reported are means ± SEM for 6-10 animals in each group. * P < 0.05 vs. untreated.

**Figure 6** Effect of sepiapterin on acetylcholine (ACh)-stimulated vasorelaxation of coronary artery segments. Vasorelaxation was examined in the absence (closed squares) and presence (open squares) of 10 µM sepiapterin. Sepiapterin treatment attenuated ACh-stimulated vasorelaxation in control diet-sedentary rats (A), while no treatment effects were observed in control diet-exercise (B), Western diet-sedentary (WD-Sed) (C), or Western diet-exercise (WD-
Ex) (D) rats. Reported are means ± SEM for 6-10 animals in each group. * P < 0.05 vs. untreated, *** P < 0.001 vs. untreated.

**Figure 7** Mean concentration-response of coronary artery segments following 3.0 µM 5-HT pre-constriction to sodium nitroprusside (SNP) stimulation (0.0001 – 1.0 µM). There were no significant differences between groups. Reported are means ± SEM for 6-11 animals in each group. CD, control diet; WD, Western diet; Ex, exercise; Sed, sedentary.

**Figure 8** Vascular tissue indices of oxidative stress measured in the thoracic aorta. NADPH stimulated H₂O₂ production was measured as an index of NADPH oxidase activity (A), which was elevated in the WD-Sed group. Thiobarbituric acid reactive substances (TBARS) were measured as an index of lipid peroxidation (B), which was not significantly elevated in any group. * P < 0.05 vs. CD-Sed, † P < 0.05 vs. WD-Ex. Reported are means ± SEM for 8-11 animals in each group. MDA, malondialdehyde; CD, control diet; WD, Western diet; Ex, exercise; Sed, sedentary.
Figure 1: Effect of Sepiapterin on 5-HT Constriction in CD and WD Mice.

A) CD-Sed: Untreated and Sepiapterin treatment show no significant difference in relaxation.

B) CD-Ex: Sepiapterin treatment significantly enhanced relaxation compared to untreated.

C) WD-Sed: Untreated and Sepiapterin treatment show no significant difference in relaxation.

D) WD-Ex: Sepiapterin treatment significantly enhanced relaxation compared to untreated.
**Table 1** Treadmill aerobic interval training protocol. Treadmill speed was changed at the indicated time point.

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**Table 2** Metabolic Parameters. Values are means ± SEM, N = 5-11 animals per group. * P < 0.05 vs. CD-Sed, † P < 0.05 vs. CD-Ex, ‡ P < 0.05 vs. WD-Ex. CD, Control diet; WD, Western diet; Sed, Sedentary; Ex, Exercise.

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<th>CD - Sed</th>
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<th>WD - Sed</th>
<th>WD - Ex</th>
<th>P-value</th>
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<tr>
<td>Body Weight (g)</td>
<td>458 ± 8.0</td>
<td>424 ± 12.5</td>
<td>499 ± 11.1†</td>
<td>456 ± 17.2</td>
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</tr>
<tr>
<td>Fat Mass (g)</td>
<td>52.0 ± 5.97</td>
<td>34.8 ± 7.48</td>
<td>76.9 ± 4.93*‡</td>
<td>44.3 ± 8.48</td>
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<tr>
<td>Lean Mass (g)</td>
<td>341 ± 6.1</td>
<td>328 ± 7.8</td>
<td>356 ± 8.3</td>
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<td>Body Fat %</td>
<td>13.1 ± 1.30</td>
<td>9.3 ± 1.82</td>
<td>17.7 ± 0.98†‡</td>
<td>10.9 ± 1.52</td>
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<td>Glucose (mg/dl)</td>
<td>107 ± 3.5</td>
<td>99 ± 1.6</td>
<td>121 ± 3.5*†</td>
<td>109 ± 3.7</td>
<td>&lt;0.001</td>
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<td>Insulin (pM)</td>
<td>125 ± 43.5</td>
<td>142 ± 28.1</td>
<td>174 ± 24.3</td>
<td>116 ± 23.4</td>
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<td>HOMA-IR</td>
<td>4.94 ± 1.84</td>
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<td>Cholesterol (mg/dl)</td>
<td>39.0 ± 4.53‡</td>
<td>34.0 ± 2.59</td>
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<td>HDL-C (mg/dl)</td>
<td>25.9 ± 2.36</td>
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<td>LDL-C (mg/dl)</td>
<td>8.6 ± 1.44</td>
<td>9.8 ± 0.66</td>
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<td>Triglycerides (mg/dl)</td>
<td>58.0 ± 9.8</td>
<td>54.8 ± 12.2</td>
<td>45.3 ± 10.0</td>
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<td>HNE-adducts (mM)</td>
<td>0.706 ± 0.07</td>
<td>0.500 ± 0.06</td>
<td>1.02 ± 0.10†‡</td>
<td>0.407 ± 0.06</td>
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Table 3  Coronary artery vasoconstriction and vasorelaxation characteristics. Values are means ± SEM, N = 6-11 animals per group.  * P < 0.05 vs. CD-Sed, § P < 0.05 vs. CD-Ex, † Significant treatment effect.  CD, Control diet; WD, Western diet, Sed, Sedentary; Ex, Exercise; ACh, acetylcholine; SNP, sodium nitroprusside; Apo, apocynin; Sep, sepiapterin.

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<td>StressKPSS (mN/mm²)</td>
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<td>2.32 ± 0.44</td>
<td>2.36 ± 0.32</td>
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<td>Stress5-HT (mN/mm²)</td>
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<td>0.204 ± 0.075</td>
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<td>ACh Eₘₐₓ</td>
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<td>93.5 ± 2.10</td>
<td>85.0 ± 3.55</td>
<td>92.6 ± 3.00</td>
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<td>SNP EC₅₀ (nM)</td>
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<td>Apo-ACh Eₘₐₓ</td>
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<td>94.3 ± 2.04</td>
<td>90.6 ± 3.03</td>
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<td>Sep-ACh Eₘₐₓ</td>
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